Study on the Extraction Technology of Ginkgo Biloba Leaf Extract by Enzymolysis Combined with Fermentation

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Abstract**—** *In this paper, we select Ginkgo biloba leaves in Taizhou as raw materials and use cellulase and pectinase to hydrolyze Ginkgo biloba leaves, and then the Ginkgo biloba leaves extract was prepared by microbial fermentation. Firstly, cellulase and pectinase were selected for single factor experiment and orthogonal experiment to determine the effect of enzyme dosage, enzymolysis time, temperature and pH value on the extraction rate of Ginkgo biloba leaves; then, microbial fermentation was used to study the effect of optimal temperature, time and pH value on the extraction rate of Ginkgo biloba leaves. The results showed that: the optimal enzyme content was 0.2%, the time of enzymolysis is 2 h, the temperature of enzymolysis was 4 ^{o}C, the pH of enzymolysis was 4.5; the optimal microorganism content of fermentation was 4%, the temperature of fermentation was 30^{o}C, the time of fermentation was 8 D, the pH of fermentation was 5， and extraction rate was 18.56%.*

Keywords— Ginkgo biloba; enzymolysis; fermentation; Ginkgo biloba extract.

I. INTRODUCTION

Ginkgo biloba is the dry leaf of Ginkgo biloba, a plant of the Ginkgo family, which likes to grow in sunny soil. Ginkgo biloba can promote blood circulation to remove blood stasis, relieve collaterals and relieve pain, and is mostly used for hyperlipidemia. Ginkgo biloba mainly has the effects of improving cardiovascular and cerebrovascular functions, anti-aging, and anti-tumor [1]. At present, the preparation of Ginkgo biloba extract mainly includes chemical methods, microwave, and enzymatic methods [2]. In order to make full use of the effective substances in Ginkgo biloba leaves, enzymatic hydrolysis is used to hydrolyze cellulose and pectin to turn large molecules into small molecules. Fermentation is used to remove toxic substances and the conditions are mild.

II. MATERIALS AND METHODS

2.1 Material

TABLE 1
MATERIAL

Materials and Reagents	Specifications	Manufacturer
Ginkgo biloba Leaf	Food Grade	Picked from Jiangsu Institute of Agriculture and Animal Husbandry Technology
Cellulase	5×10^{4}U/g	Nanning Pangbo Biological Co., Ltd.
Pectinase	3×10^{4}U/g	Nanning Pangbo Biological Co., Ltd.
S. cristatum	food grade	Nanning Pangbo Biological Co., Ltd.

2.2 Equipment and instruments

TABLE 2
EQUIPMENT AND INSTRUMENTS

Equipment and Instruments	Specifications	Manufacturer
Electronic balance	AL204 type	METTLER TOLEDO Instruments Co., Ltd.
Portable high-speed Chinese medicine grinder	FW135 type	Wenling Linda Machinery Co., Ltd.
Electric heating blast drying oven	101A-3E type	Wenling Linda Machinery Co., Ltd.
High performance liquid chromatograph	Lab Tech type	Lab Tech type Shanghai Tongwei Analytical Technology Co., Ltd.

2.3 Method

2.3.1 Technological process

Drying of Ginkgo Leaves → Smash → Enzymolysis → Fermentation → Extraction

↓

Cellulase and Pectinase

2.3.2 Operating points

Ginkgo biloba leaf pretreatment Screen the picked ginkgo leaves, rinse them with water, put them in a drying box for low-temperature drying, and then crush them with a Chinese medicine grinder, and pass through a 60-mesh sieve.

Ginkgo biloba enzymatic hydrolysis Take a certain amount of ginkgo biloba, add different amounts of enzyme (cellulase: pectinase=1:1), and add the same amount of water for enzymatic hydrolysis.

Using high performance liquid method, C18 column (5μm, 150mm×4.6mm), column temperature 25^{o}C.

2.3.3 Single factor test of ginkgo biloba enzymatic hydrolysis

The effect of the added amount of enzyme on the extraction rate of Ginkgo biloba enzymatic hydrolysis. The fixed enzymatic hydrolysis time is 2h, the enzymatic hydrolysis temperature is 45^{o}C, pH5, and the amount of added enzyme is 0.1%, 0.15%, 0.2%, 0.25%, 0.3% , 0.35%, 0.4%.

The effect of enzymolysis time on the extraction rate of Ginkgo biloba enzymatic hydrolysis. The amount of immobilized enzyme is 0.25%, the enzymatic hydrolysis temperature is 45^{o}C, and the pH is 5. The enzymatic hydrolysis time is respectively 0.5h, 1h, 1.5h, 2h, 2.5h, 3h, and 3.5h.

The effect of enzymatic hydrolysis temperature on the extraction rate of Ginkgo biloba enzymatic hydrolysis. The amount of immobilized enzyme is 0.25%, the enzymatic hydrolysis time is 2h, and the pH is 5. The enzymatic hydrolysis temperature is respectively 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C.

The effect of pH of enzymatic hydrolysis on the extraction rate of Ginkgo biloba leaves. The dosage of immobilized enzyme is 0.25%, the enzymatic hydrolysis time is 2h, the enzymatic hydrolysis temperature is 45^{o}C, and the enzymatic hydrolysis pH is 3, 3.5, 4, 4.5, 5, 5.5 , 6, 6.5 respectively.

2.3.4 Ginkgo biloba enzymatic hydrolysis response surface optimization scheme

On the basis of single factor test, select enzyme addition, enzymolysis time, enzymolysis temperature, enzymolysis pH as factors A, B, C, and D respectively to carry out a four-factor three-level response surface analysis test. The extraction rate of Ginkgo biloba flavonoids Y value, further optimize the best plan obtained by single factor, and analyze the interaction between each factor.

TABLE 3
ENZYMATIC HYDROLYSIS RESPONSE SURFACE ANALYSIS FACTORS AND LEVEL DESIGN

Level	Factor			
	A Enzyme dosage (%)	B Enzymatic hydrolysis time (h)	C Enzymolysis temperature (^{o}C)	D Enzymatic hydrolysis pH
-1	0.2	1.5	40	4
0	0.25	2	45	4.5
1	0.3	2.5	50	5

2.3.5 Enzymatic hydrolysis extraction rate algorithm

Extraction rate= Flavonoid content in filtrate/ Weight of Ginkgo biloba leaves×100%

2.3.6 Single factor test of ginkgo leaf fermentation

The influence of the amount of microorganisms on the extraction rate of Ginkgo biloba fermentation. Fixed fermentation temperature 25^{o}C, fermentation time 9d, fermentation pH5, select ginkgo biloba leaves after enzymatic hydrolysis (according to the optimal formula enzymatic hydrolysis), the amount of added microorganisms is 1 %, 2%, 3%, 4%, 5%, 6%, 7%.

The influence of fermentation temperature on the extraction rate of Ginkgo biloba fermentation. The amount of immobilized microorganisms is 4%, fermentation time is 9 days, and fermentation pH is 5. Select the ginkgo biloba after enzymatic hydrolysis (according to the optimal formula enzymatic hydrolysis), and the fermentation temperature is 10^{o}C respectively, 15^{o}C, 20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C.

The influence of fermentation time on the extraction rate of Ginkgo biloba fermentation. The amount of immobilized microorganisms is 4%, the fermentation temperature is 25^{o}C, and the fermentation pH is 5. Select the Ginkgo biloba after enzymatic hydrolysis (according to the optimal formula), and the fermentation time is 2d respectively , 4d, 6d, 8d, 10d, 12d, 14d.

The influence of fermentation pH on the extraction rate of Ginkgo biloba fermentation. The amount of immobilized microorganisms is 4%, the fermentation temperature is 25^{o}C, and the fermentation time is 8d. Select the ginkgo biloba after enzymatic hydrolysis (according to the optimal formula enzymatic hydrolysis), and the fermentation pH is 3.5, 4, 4.5, 5, 5.5, 6, 6.5.

2.3.7 Ginkgo leaf fermentation response surface optimization plan for extraction rate

On the basis of the single factor test, select the microbial addition amount, fermentation temperature, fermentation time, and fermentation pH as factors A, B, C, and D to conduct a four-factor three-level response surface analysis test. The extraction rate of ginkgo leaf flavonoids is Y value, further optimize the best plan obtained by the single factor, and analyze the interaction between the factors. The response surface analysis factors and level design are shown in Table 4.

TABLE 4
FERMENTATION RESPONSE SURFACE ANALYSIS FACTORS AND LEVEL DESIGN

Level	Factor			
	A Microbial addition (%)	B Fermentation temperature (^{o}C)	C Fermentation time (d)	D Fermentation pH
-1	3	20	7	4.5
0	4	25	8	5
1	5	30	9	5.5

2.3.8 Fermentation extraction rate algorithm

Extraction rate= Flavonoid content in filtrate/ Weight of Ginkgo biloba leaves × 100%

III. RESULTS AND ANALYSIS

3.1 Enzymatic single factor test

3.1.1 The effect of different enzymes on the extraction rate of ginkgo flavonoids

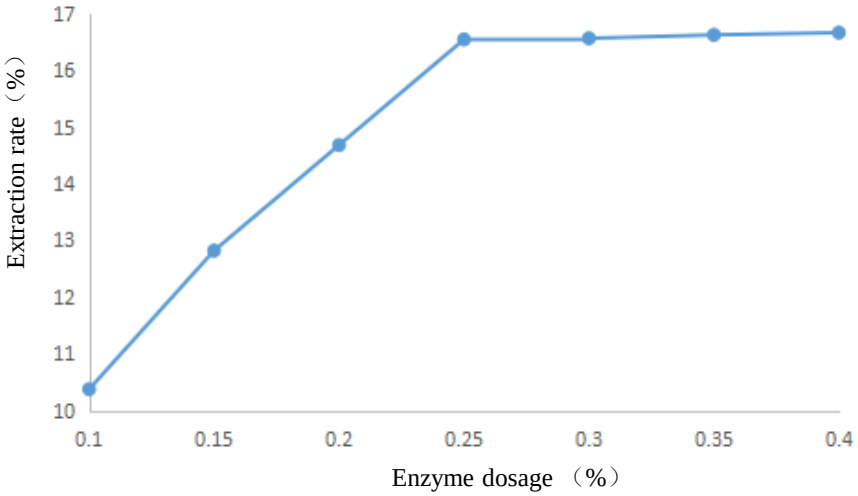

FIGURE 1: The effect of enzyme addition on the extraction rate

As the amount of enzyme increases, the extraction rate first rises and then becomes gentle. When the added amount of enzyme reaches 0.25%, the extraction rate reaches the highest (Figure 1). It may be that cellulose can degrade the cellulose skeleton of the cell wall into glucose. As the amount of enzyme increases, it will destroy the cell wall more effectively and increase the dissolution of active substances in the cell [3]. Therefore, the added amount of selected enzyme is 0.25%.

3.1.2 The effect of different enzymatic hydrolysis time on the extraction rate of ginkgo flavonoids

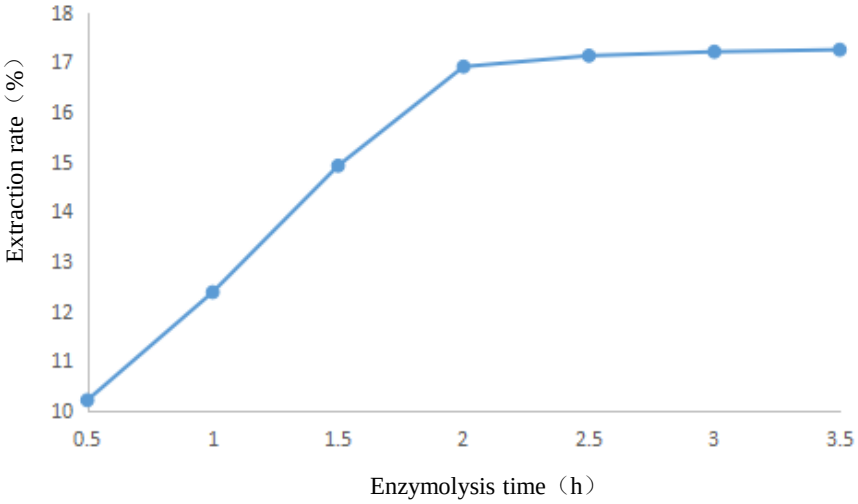

FIGURE 2: The effect of enzymolysis time on extraction rate

With the increase of enzymatic hydrolysis time, the extraction rate of ginkgo flavonoids increased significantly, and the overall trend increased first and then leveled off (Figure 2). When the enzymatic hydrolysis time is 2h, the extraction rate increases the most, and with the extension of time, the extraction rate tends to be flat. It may be that the high enzyme activity in the early stage of enzymatic hydrolysis makes the flavonoids continue to dissolve [4], and the extraction rate continues to increase, and then the time continues to increase, most of the substrates are enzymatically hydrolyzed, and the extraction rate decreases. Therefore, the optimal enzymatic hydrolysis time is 2h.

3.1.3 The effect of different enzymatic hydrolysis temperature on the extraction rate of ginkgo flavonoids

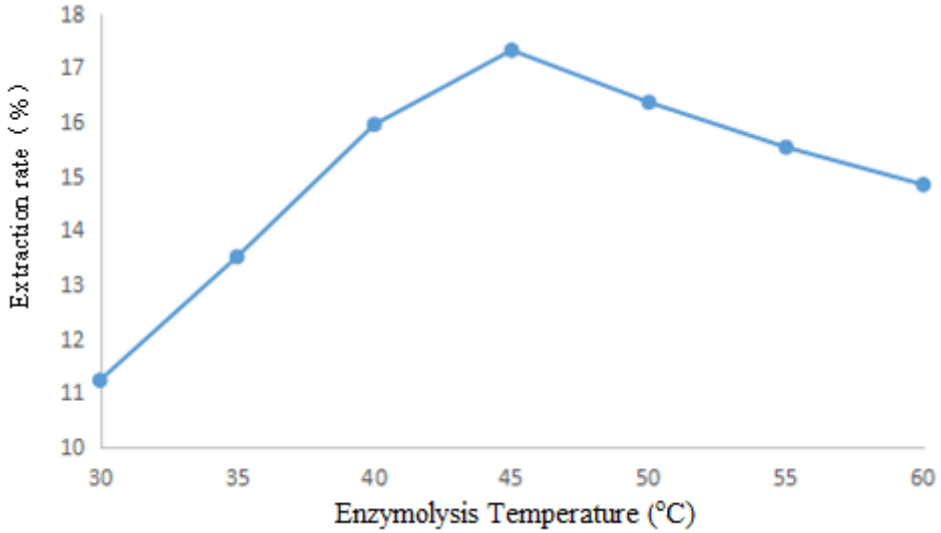

FIGURE 3: The effect of enzymatic hydrolysis temperature on the extraction rate

The temperature increases, the extraction rate first increases and then decreases (Figure 3). When the temperature reaches 45°C, the extraction rate is highest. When the temperature is between 30°C and 45°C, as the temperature increases, the enzyme activity continues to increase, causing the cell wall to continue to degrade and increase the extraction rate of ginkgo flavonoids [5]. When the temperature is between 45°C and 60°C, the temperature increases continuously and the enzyme activity decreases, thereby reducing the extraction rate of ginkgo flavonoids. Therefore, the optimal enzymolysis temperature is 45°C.

3.1.4 The effect of different enzyme hydrolysis pH on the extraction rate of ginkgo flavonoids

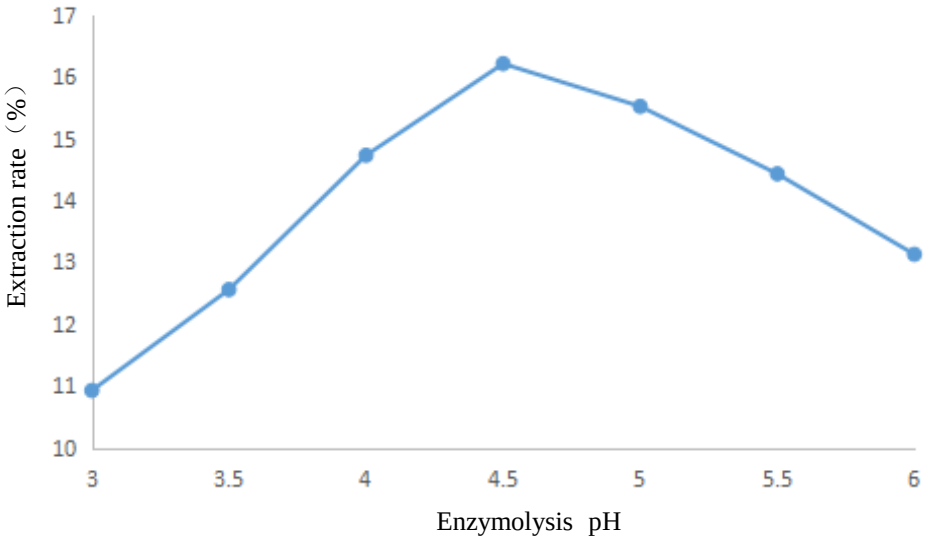

FIGURE 4: The effect of enzymatic hydrolysis pH on the extraction rate

The pH of enzymatic hydrolysis increases, the extraction rate first increases and then decreases (Figure 4). When the pH of the enzymatic hydrolysis is 4.5, the extraction rate of ginkgo flavonoids reaches the maximum. At this time, the enzymatic hydrolysis is complete, the efficiency of destroying the cell wall increases sharply, and the mass transfer resistance is reduced [6]. When the pH of enzymatic hydrolysis is in the range of 4.5-6.5, the pH of enzymatic hydrolysis continues to increase, the enzyme activity decreases, and the extraction rate of ginkgo flavonoids decreases. Therefore, choose the best enzymatic hydrolysis pH 4.5.

3.2 Enzymatic hydrolysis response surface test results

3.2.1 Response surface analysis of the extraction rate of enzymatic hydrolysis

TABLE 5
DESIGN RESULTS OF ENZYMATIC RESPONSE SURFACE TEST

Test number	Factor				Extraction rate（%）
	Enzyme addition (%)	Enzymatic hydrolysis time (h)	Enzymolysis temperature (^{o}C)	Enzymatic hydrolysis pH	
1	0.2	1.5	45	4.5	12.82
2	0.3	1.5	45	4.5	13.23
3	0.2	2.5	45	4.5	13.92
4	0.3	2.5	45	4.5	11.84
5	0.25	2	40	4	12.65
6	0.25	2	50	4	12.84
7	0.25	2	40	5	13.42
8	0.25	2	50	5	12.34
9	0.2	2	45	4	12.22
10	0.3	2	45	4	11.55
11	0.2	2	45	5	13.36
12	0.3	2	45	5	12.88
13	0.25	1.5	40	4.5	13.11
14	0.25	2.5	40	4.5	12.44
15	0.25	1.5	50	4.5	11.64
16	0.25	2.5	50	4.5	11.89
17	0.2	2	40	4.5	12.22
18	0.3	2	40	4.5	12.34
19	0.2	2	50	4.5	11.67
20	0.3	2	50	4.5	12.44
21	0.25	1.5	45	4	14.22
22	0.25	2.5	45	4	14.06
23	0.25	1.5	45	5	10.98
24	0.25	2.5	45	5	11.21
25	0.25	2	45	4.5	16.42
26	0.25	2	45	4.5	15.67
27	0.25	2	45	4.5	17.36
28	0.25	2	45	4.5	14.83
29	0.25	2	45	4.5	16.66

3.2.2 Establishment and analysis of a fitting model for the extraction rate of flavonoids from ginkgo leaves by enzymatic hydrolysis

Perform regression analysis on Table 5 to obtain the second order multiple numbers of the extraction rate (Y) and the independent variable enzyme addition amount (A), enzymolysis time (B), enzymolysis temperature (C), enzymolysis pH (D) The regression equation is:

$$Y=16.19-0.16A-0.053B-0.28C-0.28D-0.62AB+0.16AC-0.048AD+0.23BC+0.097BD-0.32CD-1.84A^2-1.73B^2-2.02C^2-1.68D^2$$

TABLE 6
ANALYSIS OF VARIANCE TABLE

Source of Variance	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	P-value	Significance
Model	59.74	14	4.27	3.68	0.0102	*
A Enzymatic hydrolysis addition amount	0.31	1	0.31	0.27	0.6128	
B Enzymatic hydrolysis time	0.034	1	0.034	0.029	0.8662	
C Enzymolysis temperature	0.94	1	0.94	0.81	0.3827	
D Enzymolysis pH	0.95	1	0.95	0.82	0.3841	
AB	1.55	1	1.55	1.34	0.2667	
AC	0.11	1	0.11	0.091	0.7671	
AD	9.03E-03	1	9.03E-03	7.79E-03	0.9309	
BC	0.21	1	0.21	0.18	0.6756	
BD	0.038	1	0.038	0.033	0.8588	
CD	0.4	1	0.4	0.35	0.5646	
A^2	21.88	1	21.88	18.89	0.0007	**
B^2	19.36	1	19.36	16.72	0.0011	**
C^2	26.54	1	26.54	22.91	0.0003	**
D^2	18.34	1	18.34	15.83	0.0014	**
Residual	16.22	14	1.16			
Lack of fit error	12.45	10	1.25	1.32	0.423	
Pure error	3.76	4	0.94			
sum	75.96	28				
R^2	0.7625					
R^2adj	0.5782					

*****Indicates extremely significant difference (P<0.01), *Indicates a significant difference (P<0.05)***

The p-value is used to test the importance of influencing factors, and its value can also reflect the interaction between influencing factors. The smaller the p-value, the more important this influencing factor is. It can be seen from Table 6 that the addition amount of enzymatic hydrolysis (A), enzymatic hydrolysis time (B), enzymatic hydrolysis temperature (C), enzymatic hydrolysis pH (D), enzymatic hydrolysis addition amount and enzymatic hydrolysis time (AB) contribute to the extraction of flavonoids from Ginkgo biloba leaves Rates have an impact, while other factors have less impact. The contribution rate is tested by the F value, and the order of the significance of each response factor to the response value is B>A>C>D, and the F value of the model is 3.68.

3.2.3 Response surface interaction of Ginkgo flavonoid extraction rate

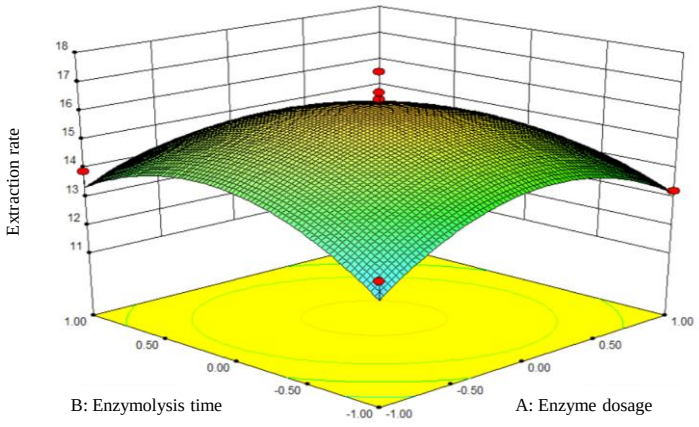

FIGURE 5: The effect of addition amount of enzymatic hydrolysis and enzymatic hydrolysis time on extraction rate

The oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious (Figure 5). The extraction rate of ginkgo flavonoids first increased and then decreased with the amount of enzymatic hydrolysis, and first increased and then decreased with the enzymatic hydrolysis time; the two factors increased in similar magnitude

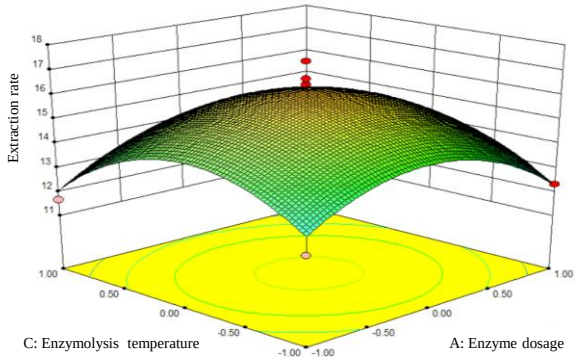

FIGURE 6: The effect of the amount of enzymatic hydrolysis and the temperature of enzymatic hydrolysis on the extraction rate

The ellipse of the graph is not obvious, indicating that the interaction of the two factors is not obvious (Figure 6). The extraction rate of ginkgo flavonoids first increased and then decreased with the amount of enzymatic hydrolysis, and first increased and then decreased with the enzymatic hydrolysis temperature; the two factors increased in similar degrees.

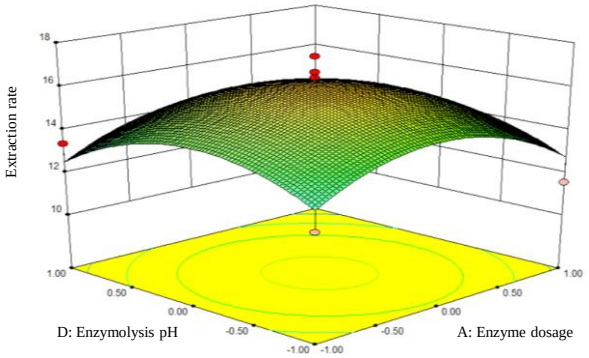

FIGURE 7: The effect of enzyme hydrolysis addition amount and enzyme hydrolysis pH on the extraction rate

The oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious (Figure 7). The extraction rate of ginkgo flavonoids increased first and then decreased with the amount of enzymatic hydrolysis, and the pH increased first and then decreased with the enzymatic hydrolysis; the two factors increased in similar ranges.

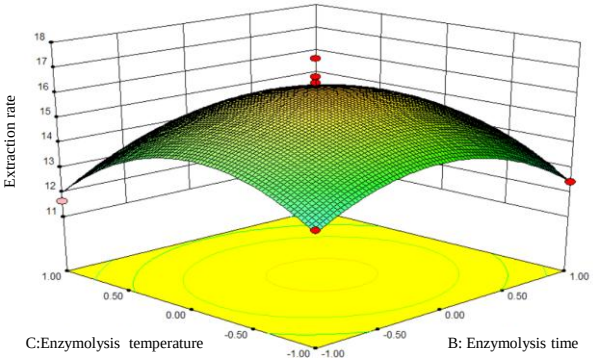

FIGURE 8: The effect of hydrolysis time and temperature on the extraction rate

The oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious (Figure 8). The extraction rate of ginkgo flavonoids first increased and then decreased with the enzymatic hydrolysis time, and first increased and then decreased with the enzymatic hydrolysis temperature; the two factors rose similarly.

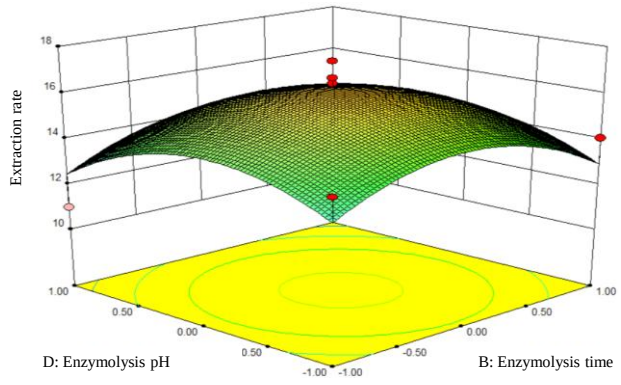

FIGURE 9: The effect of enzymolysis time and pH on the extraction rate

It can be seen from Figure 9 that the ellipse of the figure is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the enzymatic hydrolysis time, and the pH increased first and then decreased with the enzymatic hydrolysis; the two factors had similar rises.

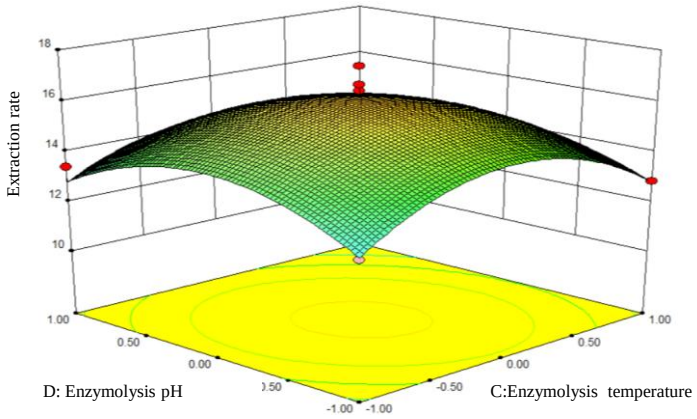

FIGURE 10: The effect of enzymolysis temperature and pH on the extraction rate

The ellipse of the figure is not obvious, indicating that the interaction of the two factors is not obvious (Figure 10). The extraction rate of ginkgo flavonoids first increased and then decreased with the enzymatic hydrolysis temperature, and the pH increased first and then decreased with the enzymatic hydrolysis; the two factors had similar rises.

According to response surface analysis, the optimal amount of enzyme addition is 0.2%, the enzymolysis time is 2h, the enzymolysis temperature is 45°C, and the enzymolysis pH is 4.5.

3.3 Fermentation single factor test

3.3.1 The effect of different microorganisms on the extraction rate of ginkgo flavonoids

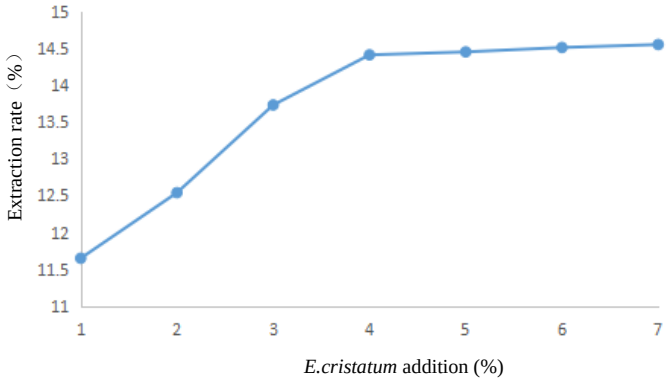

FIGURE 11: The effect of microbial addition on the extraction rate

With the increase of the amount of microorganisms added, the extraction rate shows a trend of increasing first and then gentle (Figure 11). When the amount of microorganisms added is in the range of 1% to 4%, the amount of microorganisms continues to increase and the extraction rate continues to rise. When the amount of microorganisms added was 4%, the amount of microorganisms continued to increase, and the extraction rate rose slowly and was not significant. Therefore, a 4% microbial addition was selected [7].

3.3.2 The effect of different fermentation temperature on the extraction rate of ginkgo flavonoids

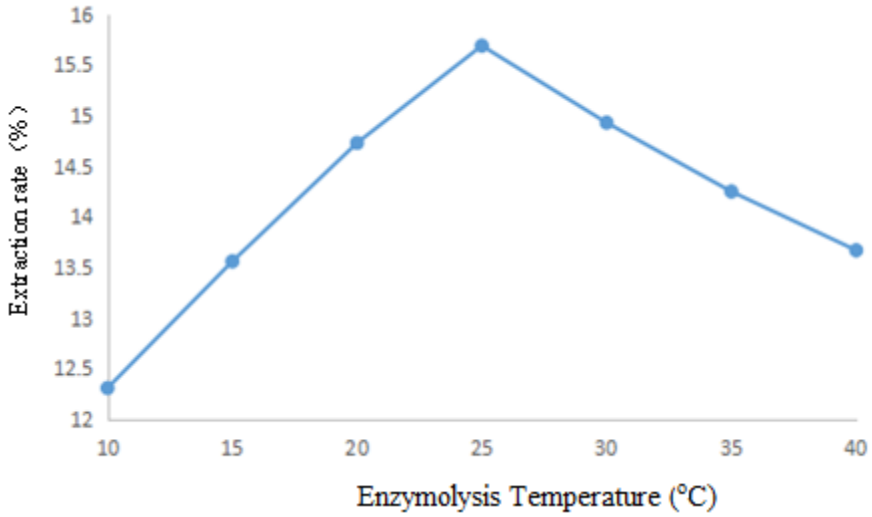

FIGURE 12: The influence of fermentation temperature on extraction rate

As the fermentation, temperature increases the extraction rate of ginkgo flavonoids increases first and then decreases. When the fermentation temperature reaches 25°C, the extraction rate is significantly higher than that at other fermentation temperatures (Figure 12). It shows that at this time, the microorganisms reach the optimum growth temperature, and increasing the temperature will hinder the growth of the microorganisms [8], and the extraction rate will decrease. Therefore, the optimal fermentation temperature was selected as 25°C.

3.3.3 The effect of different fermentation time on the extraction rate of ginkgo flavonoids

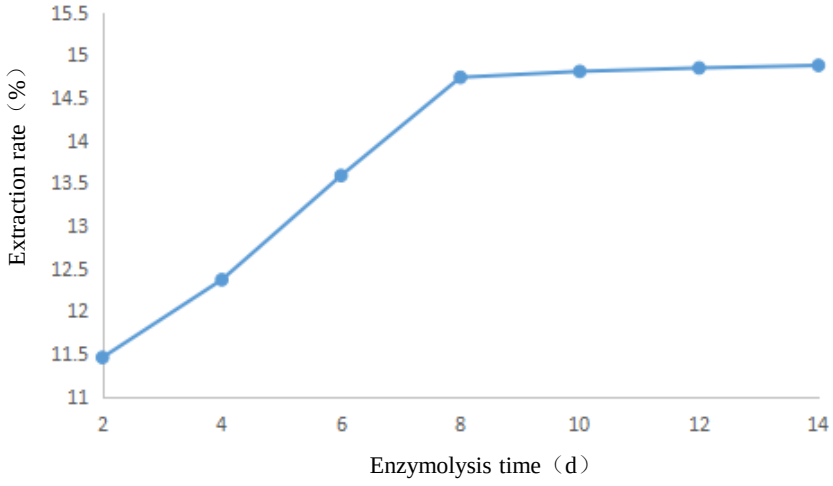

FIGURE 13: The effect of fermentation time on extraction rate

As the fermentation time continues to increase, the extraction rate of ginkgo flavonoids increases first and then becomes gentle (Figure 13). When the fermentation time is within 2-8 days, the extraction rate increases with the increase of the fermentation time, and when the fermentation time continues to increase, the extraction rate does not increase significantly. Probably because the fermentation product grew well in the early stage of fermentation [9], the extraction rate increased. Therefore, the fermentation time is 8 d.

3.3.4 The effect of different fermentation pH on the extraction rate of ginkgo flavonoids

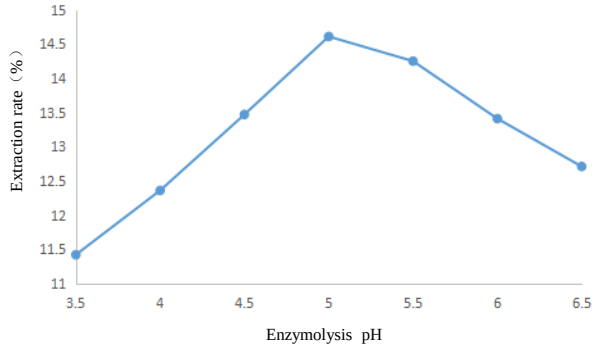

FIGURE 14: The influence of fermentation pH on extraction rate

As the fermentation pH increases, the extraction rate of ginkgo flavonoids increases first and then decreases (Figure 14). When the fermentation pH is in the range of 3.5 to 5, the extraction rate will continue to increase as the fermentation pH increases, and when the fermentation pH continues to increase, the extraction rate will continue to decrease. Probably because of the good growth environment at the initial stage of fermentation, the extraction rate continued to increase [10]. Therefore, a fermentation pH of 5 was selected.

3.4 Analysis of fermentation response surface test results

3.4.1 Response surface results of fermentation extraction rate

TABLE 7
DESIGN SCHEME AND RESULTS OF FERMENTATION RESPONSE SURFACE

Test number	Factor				Extraction rate (%)
	Enzymatic hydrolysis addition amount (%)	Fermentation Temperature (^{o}C)	Fermentation Time (d)	Fermentation pH	
1	3	20	8	5	12.78
2	5	20	8	5	13.23
3	3	30	8	5	13.92
4	5	30	8	5	11.84
5	4	25	7	4.5	12.56
6	4	25	9	4.5	12.78
7	4	25	7	5.5	13.42
8	4	25	9	5.5	12.34
9	4	25	8	4.5	12.22
10	5	25	8	4.5	11.78
11	3	25	8	5.5	13.36
12	5	25	8	5.5	12.86
13	4	20	7	5	13.21
14	4	30	7	5	12.44
15	4	20	9	5	11.64
16	4	30	9	5	11.87
17	3	25	7	5	12.22
18	5	25	7	5	12.34
19	3	25	9	5	11.86
20	5	25	9	5	12.44
21	4	20	8	4.5	14.22
22	4	30	8	4.5	14.06
23	4	20	8	5.5	11.38
24	4	30	8	5.5	11.51
25	4	25	8	5	16.32
26	4	25	8	5	15.67
27	4	25	8	5	16.63
28	4	25	8	5	14.97
29	4	25	8	5	16.16

3.4.2 Establishment and analysis of a fitting model for the extraction rate of flavonoids from fermented ginkgo leaves

Regression analysis is performed on Table 7, and the second order polynomial regression equation of extraction rate (Y) for the number of microorganisms (A), fermentation temperature (B), fermentation time (C), and fermentation pH (D) coding values is:

$$Y=15.95-0.16A-0.068B-0.27C-0.23D-0.63AB+0.12AC-0.015AD+0.25BC+0.072BD-0.32CD-1.71A^2-1.56B^2-1.93C^2-1.51D^2$$

TABLE 8
ANALYSIS OF VARIANCE TABLE

Source of Variance	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	P-value	Significance
Model	51.65	14	3.69	4.43	0.0043	**
A Enzymatic hydrolysis addition amount	0.29	1	0.29	0.35	0.5636	
B Fermentation temperature	0.056	1	0.056	0.067	0.7991	
C Fermentation time	0.89	1	0.89	1.06	0.32	
D Fermentation pH	0.63	1	0.63	0.76	0.3991	
AB	1.6	1	1.6	1.92	0.1874	
AC	0.053	1	0.053	0.064	0.8047	
AD	9.00E-04	1	9.00E-04	1.08E-03	0.9742	
BC	0.25	1	0.25	0.3	0.5924	
BD	0.021	1	0.021	0.025	0.876	
CD	0.42	1	0.42	0.51	0.4881	
A^2	19.05	1	19.05	22.87	0.0003	**
B^2	15.73	1	15.73	18.89	0.0007	**
C^2	24.16	1	24.16	29	< 0.0001	**
D^2	14.77	1	14.77	17.72	0.0009	**
Residual	11.66	14	0.83			
Lack of fit error	9.98	10	1	2.37	0.2101	
Pure error	1.68	4	0.42			
sum	63.31	28				
R^2	0.7539					
R^2adj	0.5263					

*****Indicates extremely significant difference (P<0.01), *Indicates a significant difference (P<0.05)***

Table 8 shows that the amount of microorganisms added (A), fermentation temperature (B), fermentation time (C), fermentat ion pH (D), amount of microorganisms added and fermentation time (AC), amount of microorganisms added and fermentatio n pH (AD), Fermentation temperature and fermentation time (BC) have a significant effect on the extraction rate of ginkgo fl avonoids. From the F value test of the contribution rate, the order of the significance of each response factor to the response v alue is C>D>A>B.

3.4.3 Response surface interaction of extraction rate of fermented ginkgo leaves

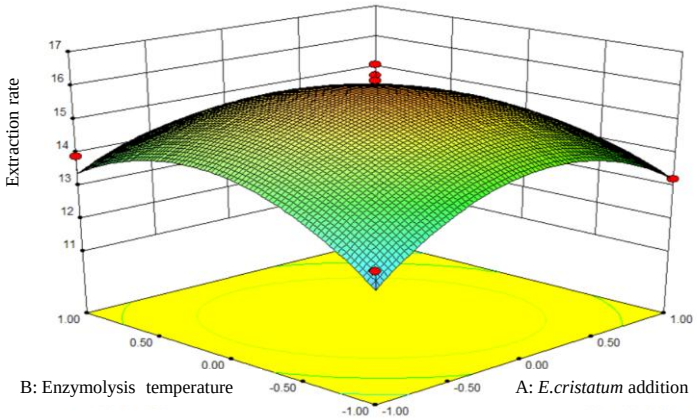

FIGURE 15: The effect of microbial addition and fermentation temperature on extraction rate

The oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the amount of microorganisms added, and first increased and then decreased with the fermentation temperature (Figure 15).

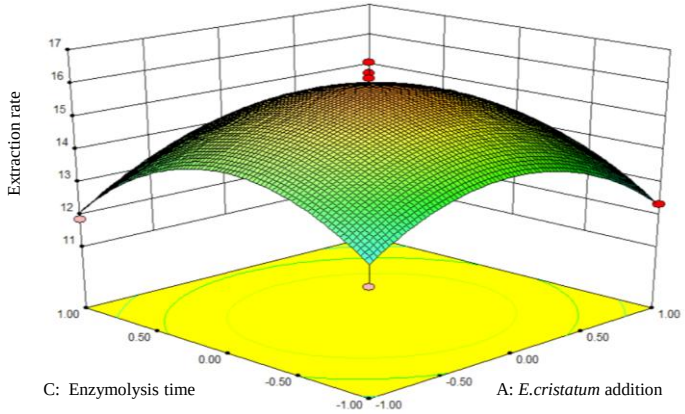

FIGURE 16: The effect of microbial addition and fermentation time on extraction rate

It can be seen from Figure 16 that the ellipse of the figure is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the amount of microorganisms added, and first increased and then decreased with the fermentation time.

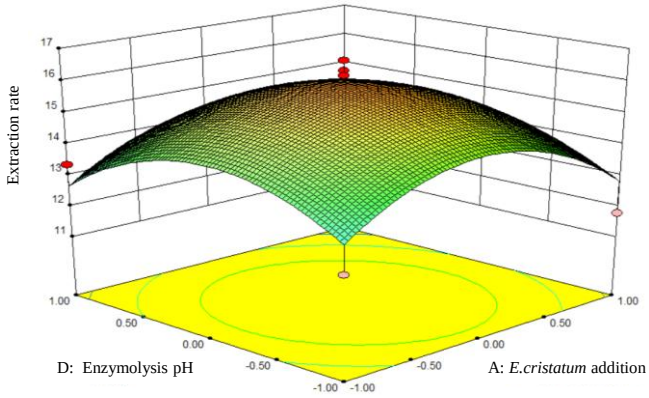

FIGURE 17: The effect of microbial addition and fermentation pH on extraction rate

It can be seen from Figure 17 that the oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the amount of microorganisms added, and the pH of the fermentation first increased and then decreased.

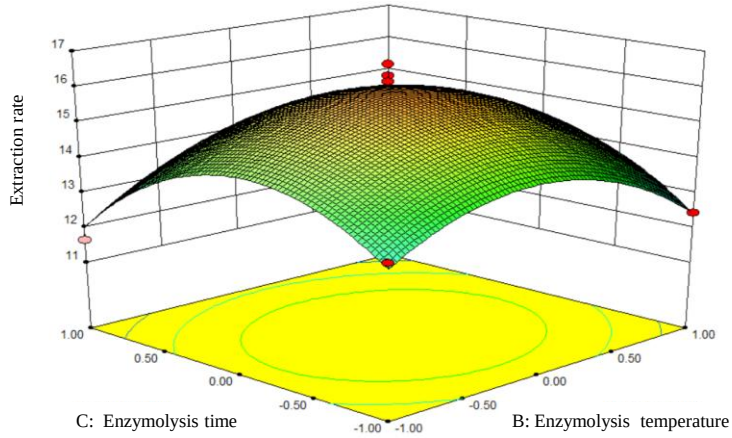

FIGURE 18: The influence of fermentation temperature and fermentation time on extraction rate

The ellipse shape of the graph is obvious, indicating that the two factors interact significantly. The extraction rate of ginkgo flavonoids first increased and then decreased with the fermentation temperature, and first increased and then decreased with the fermentation time (Figure 18).

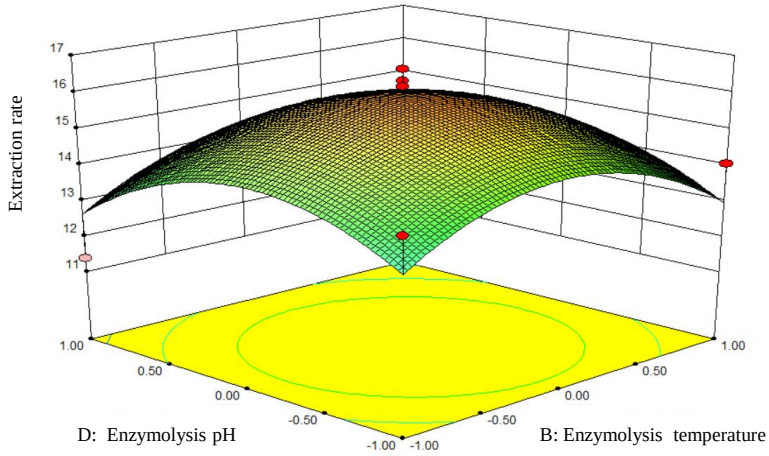

FIGURE 19: The influence of fermentation temperature and fermentation pH on extraction rate

The ellipse of the graph is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the fermentation pH, and first increased and then decreased with the fermentation temperature (Figure 19).

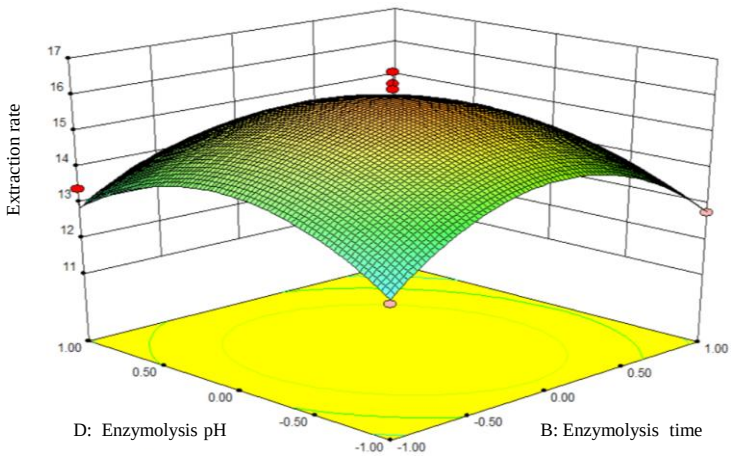

FIGURE 20: The influence of fermentation time and fermentation pH on extraction rate

It can be seen from Figure 20 that the oval shape of the graph is not obvious, indicating that the interaction of the two factors is not obvious. The extraction rate of ginkgo flavonoids first increased and then decreased with the fermentation time, and the pH increased first and then decreased with the fermentation.

Based on response surface analysis, the optimal fermentation conditions are 4% microbial addition, fermentation temperature 30^{o}C, fermentation time 8d, and fermentation pH5.

IV. CONCLUSION

In this paper, ginkgo was used as raw material, and the results of enzymatic hydrolysis and fermentation were determined according to the extraction rate through single factor experiment and orthogonal experiment of enzymatic hydrolysis and fermentation. The optimal enzyme addition amount for enzymolysis is 0.2%, the enzymolysis time is 2h, the enzymolysis temperature is 45^{o}C, and the enzymolysis pH is 4.5; the optimal microorganism addition amount for fermentation is 4%, the fermentation temperature is 30^{o}C, the fermentation time is 8d, and the fermentation pH is 5, The extraction rate was 18.56%.

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REFERENCES

[1] Shi Huijun, Wang Wenfeng, Dai Yujun. Optimization of Enzymatic Extraction Process of Ginkgo Leaf Flavonoids. Food Science an d Technology, 2014, 39(10): 208-211.

[2] Feng Zili, Zhao Zhengdong, Sun Qian, Zhu Zhibin, Li Ying, Ren Xiaofeng. Orthogonal experiment to optimize the process of extract ing ginkgo biloba flavonoids and lactones by enzymatic hydrolysis. Applied Chemical Industry, 2019, 48(05):1119 -1121.

[3] Zhang Xiaojuan, Zhao Zhengdong, Zhang Chenlu, Feng Zili, Ren Xiaofeng, Liu Jianxin. The effect of compound enzyme pretreatme nt method on the extraction rate of total flavonoids and total lactones from Ginkgo biloba leaves. Chinese Patent Medicine, 2018,40 (08):1848- 1851.

[4] Li Chao. Optimization of cellulase extraction process for total flavonoids in Ginkgo biloba. Agricultural Machinery, 2013(09): 94-97.

[5] Wu Meilin, Zhou Chunshan, Chen Longsheng, Zhong Shian, Gu Fangfang. Enzymatic extraction of ginkgo flavonoids. Research and Development of Natural Products, 2004(06):557-560.

[6] Zhang Xiaoqing, Cheng Ning, Qin Bei, Zhou Xiaohua, Chen Huiying. Enzymatic hydrolysis-solvent extraction of active ingredients from Ginkgo biloba leaves. Applied Chemical Industry, 2005(10): 48-49+56.

[7] Zhan Xin, Xin Min, Wang Ran, Tang Tao, Tang Jintian, Yue Bingfei. Comparison of in vitro antioxidant activity of extracts from Gi nkgo biloba fermentation broth in different days. Chinese Journal of Experimental Formulas, 2014, 20(03): 118-123.

[8] Lin Biaosheng, Wu Jiangwen, Jiang Huoxiang, Dai Ailing, Yang Xiaoyan. Screening of strains suitable for ginkgo biloba fermentatio n and fermentation effect. Jiangsu Agricultural Sciences, 2016, 44(04): 315-317.

[9] Zhan Xin. Bioactivity detection and separation of ginkgo biloba fermentation products and optimization of fermentation conditions [D]. Beijing University of Traditional Chinese Medicine, 2014.

[10] Yang Fang, Zhao Chongyan, Qu Qingsong, Liu Ziyao, Shi Xinyuan. Research progress in ginkgo leaf fermentation. Modern Chinese Medicine, 2018, 20(08): 1034-1038.